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Characterization of the Extracellular Lipase of *Bacillus subtilis* and Its Relationship to a Membrane-bound Lipase Found in a Mutant Strain*

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Bacillus subtilis **CMK_{33} is a mutant that is more osmotically fragile than the wild type when it is converted to the protoplast form. The protoplasts of this mutant contain a membrane-bound lipase, which is not found in protoplasts of the wild type. Hydrolysis of the membrane lipid of mutant protoplasts by the lipase is the cause of their fragility. A protein found in the wild type organism specifically inhibits the lipase (Kent, C., and Lennarz, W. J. (1972) *Proc. Natl. Acad. Sci. U. S. A.* 69, 2793–2797).**

This paper reports that cultures of both mutant and wild type cells contain an extracellular lipase which accumulates during the logarithmic phase of growth. The extracellular activity appears to be induced by a component of the growth medium. The membrane-bound lipase of the mutant has been partially purified and its properties have been compared to those of the extracellular lipase of the wild type. Their properties and sensitivity to the wild type inhibitor are similar, which suggests that the two molecules are closely related.

The subcellular location of the lipase in the mutant has been investigated and compared to the location of the membrane-bound portion of the lipase inhibitor in the wild type. The lipase is located almost exclusively in the cytoplasmic membrane and not in mesosomal vesicles. In contrast, the lipase inhibitor is located in both types of membranes and is more concentrated in mesosomal vesicles.

Under appropriate conditions, the appearance of new extracellular lipase activity in mutant cultures is paralleled by the loss of an equivalent amount of lipase activity from protoplasts prepared from the cells. This suggests that the membrane-bound lipase may be an intermediate in the secretion of the extracellular lipase. Because of the mutation in *B. subtilis* CMK_{33}, which results in the absence of the lipase inhibitor, this intermediate can be found in protoplasts of the mutant, although it is not detectable in the wild type. Consequently, the mutant may be useful in studies of the mechanism of secretion of exoenzymes by *Bacilli*.

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Bacillus subtilis CMK_{33} is a mutant that was selected because its protoplasts were more osmotically fragile than those of the parent organism, *B. subtilis* Mu8u5u5 (1). Its increased fragility was correlated with a 50 to 70% reduction in membrane lipid content that was caused by the sequential hydrolysis of the lipid by a membrane-bound lipase A_1 and a soluble lysophospholipase (2). This hydrolysis was not observed in growing mutant cells, but occurred only during the treatment with lysozyme that was used to convert the cells to protoplasts. Wild type cells contained a protein that specifically inhibited the lipase and, consequently, prevented lipid hydrolysis during the conversion of wild type cells to protoplasts. Addition of purified inhibitor to mutant cells during their conversion to protoplasts prevented hydrolysis of the membrane lipid and restored the osmotic stability of the mutant protoplasts (3). This indicated that the primary defect in the mutant was either the absence of the lipase inhibitor, or its synthesis in a defective form.

An understanding of the function of the lipase and its inhibitor at first proved elusive. Although mutant protoplasts contained a large quantity of lipase, no lipase activity could be detected in mutant cells under normal growth conditions. A phospholipase activity appeared in both mutant and wild type cells during sporulation, but its properties were different than those of the lipase from mutant protoplasts, and it was not affected by the wild type inhibitor protein (4).

The results presented in this report show that cultures of both mutant and wild type cells contain an extracellular lipase that is closely related to the membrane-bound lipase of the mutant. The extracellular lipase may function as a digestive enzyme to break down lipid micelles into fatty acids which can be transported into the cell. This hypothesis suggests that the function of the inhibitor is to protect cell membranes during and after lipase secretion. Under appropriate conditions, the membrane-bound lipase appears to be transferred from the membrane into the growth medium. This indicates that the membrane-bound lipase may be an intermediate in the secretion process.

EXPERIMENTAL PROCEDURES

Materials

All solvents were analytical reagent grade. Ribonuclease was purchased from Worthington. Phospholipase A from *Vipera russeli* and lysozyme were obtained from Sigma. Deoxyribonuclease was purchased from ICN. The nonionic detergent, Cutscum, (iso-octylphenoxypolyoxyethanol) was obtained from Fischer.

DEAE-cellulose (standard type, 0.89 meq/g) was purchased from Schleicher and Schuell, Inc. The dry powder was treated once for 30 min with 20 volumes of 0.5 N HCl, and twice for 30 min each with 20 volumes of 0.5 N NaOH. After each treatment, the cellulose was washed with distilled water until the effluent reached neutral pH. The swollen cellulose was stirred into 40 volumes of 0.005 M $MgCl_2$,

0.5 M sodium phosphate, pH 7.0, packed into a column, and equilibrated with 0.005 M $MgCl_2$, 0.05 M sodium phosphate, pH 7.0.

Determination of Radioactivity

Samples were added to 10 to 15 ml of Hydromix scintillation fluid (Yorktown Research) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Analytical Methods

Protein was determined by the method of Lowry *et al.* (5). Bovine serum albumin (Miles Laboratories, Inc.) was used as a standard. Phosphate was determined according to the procedure of Bartlett (6). Fatty ester was determined by the method of Stein and Shapiro (7). The benzyl ether of dipalmitoyl-glycerol (provided by Dr. E. Gayle Schneider, Johns Hopkins University) was used as a standard.

Bacterial Strains

The strains used in this study were *Bacillus subtilis* Mu8u5u5 (referred to as "wild type"), a derivative of strain 168 auxotrophic for threonine, leucine, and methionine (8), and *B. subtilis* CMK_{33} (referred to as "mutant") obtained by mutagenesis of *B. subtilis* Mu8u5u5 (1). Both strains were stored in 15% glycerol at −80°C.

Growth and Harvesting of Cells

Cells of wild type and mutant were grown as previously described (2) in a medium containing 1% Bacto-peptone, 1% yeast extract (Difco Laboratories), 0.5% NaCl, 0.04% Na_2HPO_4 adjusted to pH 7.0 with NaOH. Cells were incubated on a rotary shaker (340 rpm) at 30°C in 250-ml flasks containing 50- to 60-ml cultures, or 2-liter flasks containing 400- to 500-ml cultures. Cells to be used for mutant membrane preparations were grown in flasks with baffles. A 50-ml starter culture was inoculated with 2 ml of a suspension of cells in 15% glycerol and incubated with shaking for 6 to 8 h. Cultures were inoculated in the evening with 0.2 ml of the starter culture and incubated for 10 to 12 h at 30°C without shaking. Early in the morning, the shaker was activated by a timer and 2 to 5 h later the cells were harvested at mid to late logarithmic phase, when the optical density at 660 nm was 3.0, by centrifugation at 12,000 × *g* for 10 min at 4°C.

Electron Microscopy

Cells were pelleted by centrifugation at various times during the treatment with lysozyme in Mg^{2+}/sucrose buffer. The pellets were fixed in 3% glutaraldehyde in Mg^{2+}/sucrose buffer. Mesosomal and cytoplasmic membrane pellets were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2. All samples were postfixed in 2% osmium tetroxide in 0.1 M sodium phosphate, pH 7.2 (9). Specimens were dehydrated by passage through a graded acetone series and embedded in Epon 812 (10). Thin sections were stained with uranyl acetate and lead citrate (11). Preparations were examined in a Siemens Elmiskop 1 double-condenser electron microscope at 60 kV at field magnification ranging from 12,000 to 20,000.

Enzyme Assays

Lipase activity was determined by measuring the release of ^3H-fatty acid from [1-*acyl*-^3H]phosphatidylethanolamine (7.5×10^4 to 1.5×10^6 cpm/μmol). The enzyme solution (5 to 50 μl) was added to 15 μl of 3 M KCl, 5 μl 0.02 M $CaCl_2$, and 0 to 45 μl of distilled water. When cells or protoplasts were assayed, the assay mixture contained 0.6 M sucrose. Sucrose has no effect on the activity of purified lipase. The substrate was dispersed in 0.167 M Tris/maleate, pH 8.0, 2.0% Cutscum (Fisher) by three 10-s periods of sonication (Biosonik sonic oscillator, small probe, 20% maximum intensity). The assay was initiated by addition of 30 μl of this dispersion. Incubations were performed at 30°C for 5 to 30 min and terminated by the addition of 3.3 ml of isopropyl alcohol/heptane/water/1 N H_2SO_4 (40:10:5.1:1). The solutions were mixed thoroughly and incubated for 10 min at room temperature. The ^3H-fatty acids were extracted as described (12). A unit of lipase activity is the amount that catalyzes the release of 1 μmol of fatty acid/min.

Inhibitor activity was assayed as described (3). One inhibitor unit is defined as the amount of inhibitor needed to reduce the activity of the membrane-bound lipase of the mutant (20 μg of protein) by 30%.

Succinate dehydrogenase activity was measured by the method of Ferrandes *et al.* (13). Measurements were made at room temperature with a Beckman model DU spectrophotometer attached to a Gilford model 2000 multiple sample absorbance recorder. A unit of succinate dehydrogenase is the amount that catalyzes the reduction of 1 μmol of dichlorophenolindophenol (in the presence of succinate) per min.

Cell Fractionation

Mutant and wild type cells were fractionated by the following adaptations of the method of Ferrandes *et al.* (13).

Fractionation of Mutant Cells—Mutant cells (400-ml culture, about 2 g wet weight) were grown and harvested as described above. The cell pellet was washed once with 60 ml of sucrose buffer (0.6 M sucrose, 0.05 M sodium phosphate, pH 7.0, 4°C) containing 0.01 M EGTA[1] (Sigma) and resuspended in 4 ml of sucrose buffer containing 0.01 M EGTA and 0.025 M $MgCl_2$. The cells were converted to protoplasts by incubation with 18 mg of lysozyme for 30 min at 30°C. The protoplasts were sedimented by centrifugation at 10,000 × *g* for 20 min at room temperature. The supernatant fluid, containing soluble periplasmic molecules and mesosomal membranes, was removed and the mesosomes were sedimented by centrifugation at 100,000 × *g* for 1 h. The mesosomal pellet was washed once in 5 ml 0.01 M sodium phosphate, pH 7.0, and resuspended in 0.3 ml of the same buffer. The protoplast pellet was resuspended in 10 ml of Mg^{2+}/phosphate buffer (0.05 M sodium phosphate, 0.005 M $MgCl_2$, pH 7.0) containing 1 mg of DNase and 1.2 mg of RNase. The suspension of lysed protoplasts was stirred for 30 min at 0°C, then the cytoplasmic membranes were sedimented by centrifugation for 1 h at 100,000 × *g*. The membrane pellet was washed once with 10 ml of 0.01 M sodium phosphate, pH 7.0, and resuspended in 0.8 ml of the same buffer.

Fractionation of Wild Type Cells—Wild type cells (2-liter culture, about 4 g wet weight) were grown and harvested as described above. The cells were washed with 400 ml of sucrose buffer containing 0.015 M $MgCl_2$ and resuspended in 20 ml of the same buffer containing 88 mg of lysozyme. Protoplast formation and fractionation was carried out as described for mutant membranes, except for the following modifications. The mesosomal pellet was suspended in 0.8 ml of 0.01 M sodium phosphate, pH 7.0, without washing. The protoplast pellet was suspended in 20 ml of sucrose buffer and added to 260 ml of Mg^{2+}/phosphate buffer containing 6 mg of DNase and 6.6 mg of RNase. After a 30-min incubation at 0°C, cytoplasmic membranes were sedimented by centrifugation at 80,000 × *g* for 3 h, and suspended in 8 ml of 0.01 M sodium phosphate, pH 7.0, without washing.

The mesosomal and cytoplasmic membranes of the wild type were further purified by sucrose density gradient centrifugation. Each fraction was sedimented through a discontinuous gradient of sucrose (density = 1.10; 1.15; 1.20; 1.25) in 0.05 M sodium phosphate, pH 7.0. One milliliter of each membrane suspension (11.6 mg of mesosomes, 20.5 mg of cytoplasmic membranes) was applied to a 15-ml gradient (3.75 ml of each sucrose solution). The gradients were centrifuged for 4 h at 70,000 × *g*. Bands at each interface were collected with a pipette, diluted approximately 2-fold with 0.05 M sodium phosphate, pH 7.0, and centrifuged for 2 h at 100,000 × *g*. The pellets were resuspended in 0.1 to 0.3 ml of 0.01 M sodium phosphate, pH 7.0. Bands from the gradients are referred to, in order of increasing density, as 1M, 2M, 3M, 4M (from the mesosomal membrane fraction) and 1C, 2C, 3C, 4C (from the cytoplasmic membrane fraction). In agreement with the results of Ferrandes *et al.* (13), band 2M contained the most highly purified mesosomal membranes and band 3C contained the most highly purified cytoplasmic membranes.

Partial Purification of the Membrane-bound Lipase of the Mutant

Step A: Preparation of the Membrane Fraction—Membranes of mutant cells were prepared as described previously (2) with the following modifications. The growth medium contained 2% Bacto-peptone, 2% yeast extract, 1% NaCl, 0.04% Na_2HPO_4, pH 7.0. This is twice the concentration of the major components used in earlier work (see Fig. 4). Cells from a 7.5-liter culture were washed with 1.2 liters of Mg^{2+}/sucrose buffer (0.6 M sucrose, 0.05 M sodium phosphate, pH 7.0, 0.005 M $MgCl_2$). To convert the cells to protoplasts, the cells were resuspended in 75 ml of Mg^{2+}/sucrose buffer containing 440 mg of lysozyme and incubated for 30 min at 30°C. The protoplasts were added, with stirring, to 1.2 liters of Mg^{2+}/phosphate buffer containing 12 mg of DNase and 13 mg of RNase. After a 30-min incubation at 0°C, the membranes were sedimented by centrifugation for 1.5 h at

[1] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid.

27,000 × *g*. The membrane pellet was washed twice by suspension in 300 ml of Mg^{2+}/phosphate buffer and centrifugation at 80,000 × *g* for 45 min. The pellet was washed once more in 0.01 M sodium phosphate, pH 7.0, resuspended in 12 ml of the same buffer, and stored at −20°C.

Step B: Base Wash—The membranes (460 mg of protein) were thawed, brought to a protein concentration of 12 mg/ml with distilled water, dialyzed against 2 liters of 0.003 N NaOH (pH 10.6) for 1 h at 0°C, and sedimented by centrifugation for 90 min at 100,000 × *g*.

Step C: Solubilization by Urea—The membrane pellet from the base wash (311 mg of protein) was resuspended in 9.9 ml of 0.05 M sodium phosphate, pH 7.0. Distilled water and 10 M urea (J. T. Baker Chemical Co., purified by the method of Duesberg *et al.* (14)) were added to bring the final solution to a concentration of 3 M urea and 0.01 M sodium phosphate. The suspension was stirred slowly for 1 h at 0°C. Membranes were sedimented by centrifugation for 90 min at 100,000 × *g*. The supernatant fluid (46 ml), containing solubilized lipase, was removed and dialyzed overnight against 15 liters of Mg^{2+}/phosphate buffer (4°C).

Step D: DEAE-cellulose Chromatography—The solubilized enzyme preparation (22.5 mg of protein) was applied to a DEAE-cellulose column (1.7 × 5.5 cm) equilibrated with Mg^{2+}/phosphate buffer. The column was washed with 20 ml of 0.01 M KCl in Mg^{2+}/phosphate buffer, then with 50 ml of 0.25 M KCl in the same buffer. The major portion of lipase activity was eluted with 0.4 M KCl in Mg^{2+}/phosphate buffer. The column was washed with this solution until no further lipase appeared in the eluate. This fraction (28.5 ml) was dialyzed overnight against 4 liters of 0.01 M sodium phosphate, pH 7.0, (4°C). After dialysis the solution was divided into 0.5-ml aliquots and stored at −20°C.

Preparation of the Lipase from the Growth Medium of the Wild Type

For determination of optimal assay conditions and substrate specificity, the lipase from growth medium was prepared by the following procedure. A wild type culture (2 liters) was grown as described above in 3-fold concentrated growth medium (see Fig. 4). To inhibit protease activity, the culture was brought to a concentration of 0.001 M α-toluenesulfonyl fluoride (Eastman Kodak Co.) by addition of 2 ml of 0.1 M α-toluenesulfonlyl fluoride in ethanol. The cells were harvested by centrifugation and an additional 2 ml of the α-toluenesulfonyl fluoride solution was added to the supernatant growth medium. The lipase activity of the medium (33 units) was concentrated 3-fold in a model 2000 Amicon apparatus fitted with a UM-10 filter, then an additional 13-fold in a model 402 Amicon apparatus with a PM-30 filter. The concentrated medium, which contained all of the recovered activity (18 units), was then centrifuged at 100,000 × *g* for 2 h. The supernatant (43 ml, 18 units) was stored at −20°C and was stable for several weeks.

For determination of the sensitivity of the growth medium lipase to inhibitor, the lipase was prepared as follows. A wild type culture (2.5 liters) was grown in 2-fold concentrated growth medium and the cells were harvested as usual. α-Toluenesulfonyl fluoride was added to the culture and to the supernatant growth medium as described above. The lipase activity of the medium (18 units) was concentrated 80-fold with a 49% recovery in an Amicon apparatus fitted with a PM-30 filter. The concentrated medium (25 ml, 7.9 lipase units) was applied to a column of Sephadex G-200 (2.5 × 58 cm) equilibrated with 0.01 M sodium phosphate (pH 7.0), 0.001 M EDTA, and 0.001 M α-toluenesulfonyl fluoride. The total recovery of activity, all of which was eluted in the void volume, was 19% of the activity in the column sample. The lipase solution was centrifuged at 100,000 × *g* for 2 h. The supernatant (43 ml, 1.5 lipase units), which contained all of the lipase activity, was concentrated to 7 ml in an Amicon apparatus fitted with a PM-30 filter. The concentrated solution (19.6 mg, 1.0 lipase units) contained 5% of the lipase activity that was present in fresh growth medium. The specific activity of the lipase was 11-fold higher than that of growth medium. The solution was stored at −20°C and was stable for several weeks.

Thin Layer Chromatography

Plates for thin layer chromatography (0.4 or 0.7 mm thick) were prepared with a suspension of 40 g of Silica Gel H (EM Laboratories, Inc.) in 95 ml of 0.001 M sodium borate. Chromatograms of phospholipids and diglucosyl diglyceride were developed in the following solvent systems: A (neutral), chloroform/methanol/H_2O (90:40:5); B (basic), chloroform/methanol/1 M NH_4OH (80:36:2); C (acidic), chloroform/methanol/acetic acid (65:25:8). Chromatograms of diglyceride were developed in solvent system D: petroleum ether/diethyl ether/acetic acid (80:75:1.5).

Lipid standards were obtained from the following sources: bacterial phosphatidylethanolamine and oleic acid, Applied Sciences; diglyceride and phosphatidylglycerol, Serdary Laboratories; *B. subtilis* diglucosyl diglyceride, Dr. Sharon S. Krag, Johns Hopkins University; and diphosphatidylglycerol, Sylvanna Chemical Co.

Lipid spots were visualized by placing the plate in iodine vapor for 5 to 10 min. After the spots were marked, iodine was removed from the plates by heating them at 100°C for 10 to 20 min. Phospholipids were detected with a molybdenum blue spray (15). Lipids containing amino groups were detected with ninhydrin spray (15). Diglucosyl diglyceride was visualized with an aniline/diphenylamine spray (16).

To measure radioactivity in lipid spots, silica gel zones were scraped from the plates, transferred to vials, and counted after addition of 0.5 ml of methanol/H_2O (1:1) and 10 ml of Hydromix scintillation fluid.

To recover lipids silica gel zones were scraped from the plates into 40-ml conical tubes. Phospholipids and diglucosyl diglyceride were eluted by washing twice with 3 volumes of chloroform/methanol/ammonium hydroxide/H_2O (20:20:1:1). Diglyceride was eluted with chloroform/methanol/H_2O (10:10:1). Chloroform was added to the lipid solutions to bring the final proportion to chloroform/methanol (2:1). This solution was washed with 0.2 volume of 0.9% saline (17). The chloroform layer was evaporated to dryness under reduced pressure, the wash procedure was repeated once, the solvent was evaporated, and the lipid was dissolved in a small volume of chloroform for storage at −20°C.

Preparation of Lipids

[1-*acyl*-^{3}H]Phosphatidylethanolamine (3×10^5 cpm/nmol) was prepared as previously described (18) using [^{3}H]oleic acid (New England Nuclear) and bacterial phosphatidylethanolamine (Applied Science Laboratories, Inc.). The specific activity was adjusted by the addition of either the unlabeled bacterial phosphatidylethanolamine or *Escherichia coli* phosphatidylethanolamine provided by Dr. E. Gayle Schneider.

For preparation of *B. subtilis* lipids, wild type cells (23.5 g wet weight) were grown and harvested as described above. Lipids were extracted at room temperature with acidic chloroform/methanol (2:1) as previously described (2).

The lipid extract (120 μmol of lipid phosphorus) was fractionated by DEAE-cellulose chromatography according to an adaptation of the method of Rouser *et al.* (19). The sample was applied to a 300-ml DEAE-cellulose column in 20 ml of chloroform. The lipids were eluted sequentially with 5 column volumes each of: 1, chloroform; 2, chloroform/methanol (7:1); 3, chloroform/methanol (7:3); 4, chloroform/methanol/ammonium hydroxide/0.5 M ammonium acetate, (40:10:1:0.5).

Fraction 1 contained diglyceride and other nonpolar lipids. Diglyceride was purified by thin layer chromatography in solvent system D. The purified product (25.5 μmol) contained less than 0.003 μmol of phosphate/μmol of ester. It ran as a single spot in solvent system A and two narrowly resolved spots in solvent system D. The spots have been identified as the 1,2- and 1,3-isomers of diglyceride (20). Each of the two isomers was purified in solvent system D immediately before their use in enzyme assays.

Fraction 2 contained diglucosyl diglyceride and lysylphosphatidylglycerol. These two lipids were separated by thin layer chromatography in solvent system B. Purified lysylphosphatidylglycerol contained significant amounts of phosphatidylglycerol and free lysine after storage for 1 day. Purified diglucosyl diglyceride (5.8 μmol) ran as a single hexose-positive spot in solvent systems A and B.

Fraction 3 contained phosphatidylethanolamine and a small amount of lysophosphatidylethanolamine. Phosphatidylethanolamine was purified by thin layer chromatography in solvent system A. The purified product (14.4 μmol) ran as a single amine-positive spot in solvent systems A and C.

Fraction 4 contained phosphatidylglycerol, diphosphatidylglycerol, and a small amount of free fatty acid. Phosphatidylglycerol was purified by thin layer chromatography in solvent system A. The purified lipid (60 μmol) ran as a single phosphate-positive spot in solvent system A. After chromatography in solvent system C, the phosphate-positive spot showed a slight reaction with ninhydrin at its lower end, indicating some contamination with phosphatidylethanolamine. Diphosphatidylglycerol was purified by thin layer chromatography in solvent system A. The purified product (2.1 μmol) ran as a single phosphate-positive spot in solvent systems A and B.

For preparation of radioactive lipids, fourteen 50-ml cultures of wild type cells were grown in the CH/S medium described by Pollock *et al.* (21), containing 0.6% Casamino acids. Each culture was supplemented with 200 μCi of [^{3}H]isoleucine and 200 μCi of [^{3}H]leucine (30 to 60 Ci/mmol, New England Nuclear). This label is incorporated into the branched chain fatty acids of *B. subtilis* (22). Lipids were extracted and fractionated by DEAE-cellulose and thin layer chromatography as described above. Purified [^{3}H]phosphatidylglycerol was fractionated a second time by DEAE-cellulose chromatography to remove any traces of [^{3}H]phosphatidylethanolamine.

[^{3}H]Diglyceride (5.8 μmol, 1.3×10^6 cpm/μmol) had a radiochemical purity of 98 to 99% as determined by thin layer chromatography in solvent systems A and D. About 25% of the tritium was in the 1,3-isomer and 75% in the 1,2-isomer. These isomers were separated, as described above, immediately before use in enzyme assays. Purified 1,2-[^{3}H]diglyceride contained 2% 1,3-[^{3}H]diglyceride and purified 1,3-[^{3}H]diglyceride contained 13% 1,2-[^{3}H]diglyceride. [^{3}H]Diglucosyl diglyceride (1.9 μmol, 1×10^6 cpm/μmol) was shown to have a radiochemical purity of 95 to 96% by thin layer chromatography in solvent systems A and B. [^{3}H]Phosphatidylethanolamine (1.2 μmol, 8.3×10^5 cpm/μmol) had a radiochemical purity of 79 to 80% as determined by thin layer chromatography in solvent systems A and C. About 7% of the tritium ran with free fatty acid in solvent system C. Hydrolysis of the [^{3}H]phosphatidylethanolamine by *Vipera russelli* phospholipase A_2 (18) showed that 39% of the tritium was in the 1-fatty acid and 61% in the 2-fatty acid. [^{3}H]Phosphatidylglycerol (6.2 μmol, 1.5×10^6 cpm/μmol) was shown to have a radiochemical purity of 92 to 96% by thin layer chromatography in solvent systems A and C. Hydrolysis by phospholipase A_2 showed that 33% of the tritium was in the 1-fatty acid and 67% in the 2-fatty acid. [^{3}H]Diphosphatidylglycerol (0.22 μmol, 3.1×10^6 cpm/μmol) had a radiochemical purity of 92 to 94% as determined by thin layer chromatography in solvent systems A and C. Since diphosphatidylglycerol is synthesized from phosphatidylglycerol (3) and its specific acivity per diacylglycerol moiety was the same as that of phosphatidylglycerol, it was assumed that the distribution of tritium between the 1- and 2-acyl positions was the same for the two lipids.

RESULTS

Extracellular Lipase—One approach to understanding the function of the lipase-inhibitor system in *B. subtilis* had been to look for conditions under which turnover or hydrolysis of membrane lipid occurred in unbroken cells. Because attempts to find such conditions were unsuccessful, it seemed possible that cell cultures might contain lipase that was not exposed to the lipid of the cell membrane. To test this possibility, lipase activity was measured in intact cells, which we thought might have lipase bound to the surface of the cell wall, and also in protoplasts and growth medium from cultures of mutant and wild type cells, using radioactive substrate of high specific activity (Table I). Very little lipase activity was expressed in intact cells of either organism. After removal of the cell wall by lysozyme, the lipase activity of mutant cells increased by 2 orders of magnitude, while that of wild type cells remained unchanged. Similar results were obtained when lipase activity was assayed by measuring hydrolysis of endogenous membrane lipid (2). The growth medium of both mutant and wild type cells contained a small amount of lipase activity. This activity was dependent on Ca^{2+} and abolished by incubation with the wild type lipase inhibitor (data not shown). Both of these characteristics suggested that the extracellular lipase might be related to the membrane lipase of the mutant.

During the course of these experiments, we observed that the amount of lipase in the culture medium was much greater if the cells were grown in concentrated medium (lower half of Table I). This was not due to the presence of lipase activity in the growth medium itself because fresh growth medium had no detectable lipase activity. The quantity of lipase in both protoplasts and medium from cell cultures depended on the concentrations of compounds of the initial growth medium as is shown in Fig. 1.

Two observations indicated that the extracellular enzyme was secreted by a normal physiological process and was not simply the result of lysis of a portion of the cells. First, when cultures were grown in enriched medium, the amount of extracellular enzyme was greater than the amount contained in protoplasts. The amount of extracellular enzyme could be accounted for by its release from cells during autolysis only if most of the cells had lysed. However, the optical density of

TABLE I

Distribution of lipase activity in mutant and wild type cultures grown in standard and enriched media

Sixty-milliliter cultures of mutant and wild type cells were grown to late logarithmic phase and the cells were harvested by centrifugation as described under "Experimental Procedures." One pair of cultures was grown in standard medium, containing 1% Bacto-peptone and 1% yeast extract as described under "Experimental Procedures." The other pair was grown in the same medium, except that the concentrations of Bacto-peptone and yeast extract were increased to 3%. The supernatant growth media from the cultures grown in standard medium were concentrated in an Amicon Diaflo apparatus fitted with a UM-10 filter. (This procedure was necessary to detect extracellular lipase activity.) The enriched media were not concentrated. The cell pellets were washed with 8 ml of Mg^{2+}/sucrose buffer and resuspended in 0.5 ml. A portion of each of the suspensions of cells grown in standard medium was assayed without further treatment. The cells were converted to protoplasts by incubation with lysozyme for 30 min at 30°C. Lipase assays were performed as described under "Experimental Procedures." Activity in all fractions was linear with time and protein concentration.

Medium	Fraction	Total activity	
		Mutant	Wild type
Standard	Cells	0.003 (1)[a]	0.005 (1.7)
	Protoplasts	0.49 (160)	0.006 (2)
	Growth medium	0.021 (7)	0.090 (30)
Enriched	Protoplasts	0.73 (240)	<0.001 (0.3)
	Growth medium	1.1 (370)	1.3 (430)

[a] One unit of activity is defined as the amount of enzyme catalyzing formation of 1 μmol of product/min. Numbers in parentheses are normalized to a value of 1 for mutant cells.

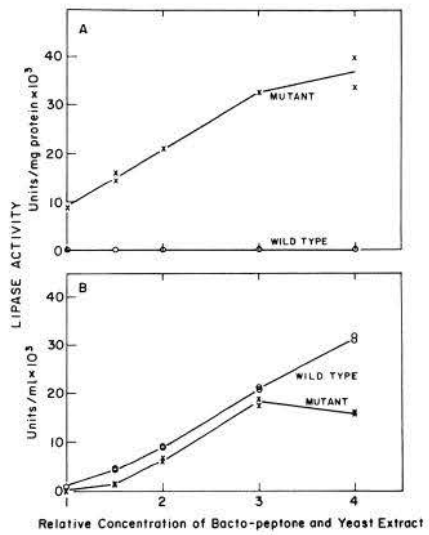

FIG. 1. Effect of growth medium on lipase activity in mutant and wild type cultures. Growth conditions were as described in "Experimental Procedures," except for the indicated variable. A relative concentration of 1 is 1% Bacto-peptone and 1% yeast extract. A unit of lipase activity is defined as the amount of enzyme catalyzing formation of 1 μmol of product/min. *A*, specific activity of lipase in protoplasts prepared and assayed as described under "Experimental Procedures"; *B*, concentration of lipase activity in growth medium assayed as described under "Experimental Procedures." O——O, wild type; ×——×, mutant.

the cultures never decreased throughout the experiments, thus indicating that massive lysis was not occurring. Second, the time course of appearance of extracellular lipase indicated that the lipase was secreted in a normal physiological way (Fig. 2). The enzyme began to accumulate during the logarithmic phase of growth and reached a peak at late logarithmic phase. The activity then declined during stationary phase. This decline was presumably caused either by gradual denaturation of the enzyme or by degradation of the enzyme by some other component of the medium. If the cells were harvested at late logarithmic phase and the clarified medium was returned to 30°C and aerated, the rate of decrease of lipase activity in the medium was the same as it was if the cells were present. This suggests that secretion of lipase by the cells does not contribute to the equilibrium level of lipase in the medium at the later stages of growth. Thus, the production of extracellular lipase paralleled growth and ceased during stationary phase when the rate of autolysis rises.

In cultures grown in standard medium, the medium of the mutant contained only one-quarter as much lipase as the medium of the wild type. Even in cultures grown in concentrated medium, extracellular lipase never reached as high a level in mutant as in wild type cultures (Fig. 1). It is not known if this property of the mutant is related to the absence of the lipase inhibitor.

The increased amount of extracellular lipase seemed to depend on the concentration of unknown high molecular weight components of the medium (data not shown). A high concentration of either Bacto-peptone or yeast extract alone could increase extracellular activity; yeast extract was the more potent of the two. As increasing amounts of macromolecules were filtered out of the concentrated medium with a series of Amicon filters of decreasing pore size, the ability of the medium to increase extracellular lipase decreased. The high molecular weight material removed by the filter could not be replaced by a mixture of lipids from *E. coli*. The enzyme was not secreted when the cells were grown in Casamino acids medium (21), in Casamino acids supplemented with 4 mg/ml of ovalbumin, or in minimal medium (1) with either glucose or glycerol as the carbon source (data not shown).

Partial Purification of Lipase from Membranes of the Mutant—The procedure for solubilization and partial purification of the membrane-bound lipase of the mutant is described under "Experimental Procedures" and summarized in Table II. In the first step (Step A under "Partial Purification of Membrane-bound Lipase of Mutants"), membranes were prepared from protoplasts by osmotic lysis, followed by three washes. Under the lysis conditions described, an average of 45% of the activity of the protoplasts was recovered in the membranes and 25% in the soluble fraction. An additional 5 to 10% was lost from the membranes during each wash. The membranes prepared by this procedure contained 35 to 40% of the lipase activity of the protoplasts and their specific activity increased 5-fold. After treatment with base (Step B), the membrane pellet contained an average of 70% of the protein and 83% of the lipase activity of the membranes prepared in Step A. The base wash was included because it resulted in a 2-fold greater purification during the subsequent solubilization step. In Step C, the lipase was solubilized by treatment of the membranes with 3 M urea followed by centrifugation. The dialyzed supernatant contained an average of 3% of the lipid phosphate, 11% of the protein, and 33% of the lipase activity of the base-washed membranes. All of the recovered activity was in the supernatant. The ratio of micromoles of lipid phosphate to mg of protein in this supernatant was 0.02 to 0.03, which is one-fifth to one-third of the ratio in mutant membranes (23). After removal of the urea, the lipase could not be sedimented by centrifugation at 100,000 × *g* for 3 h. The urea supernatant was applied to a DEAE-cellulose column (Step D). The first two washes eluted about 30% of the protein and no lipase activity. The 0.25 M KCl wash contained an average of 27% of the protein and 13% of the initial lipase activity, while the final eluate (0.4 M KCl) contained an average of 7% of the protein and 40% of the initial lipase activity. The total recovery of protein from the column averaged 65% and recovery of lipase activity averaged 60%. The overall purification of the lipase was 100- to 200-fold, with a yield of 3 to 6%.

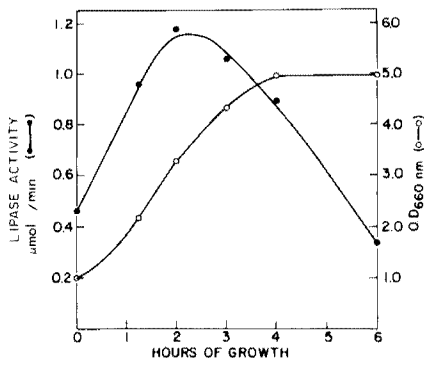

FIG. 2. Appearance of lipase activity in wild type cultures during growth. One liter of growth medium was inoculated with wild type cells as described under "Experimental Procedures." A 30-ml aliquot of the culture was taken when it reached an optical density of 1.0 and at the time points indicated. The cells in each aliquot were harvested as usual. The clarified growth medium from each aliquot was concentrated to approximately 4 ml with an Amicon Diaflo apparatus fitted with a UM-10 filter. Lipase activity was measured as described under "Experimental Procedures." ●——●, enzyme activity in growth medium; ○——○, optical density of the culture.

TABLE II

Partial purification of the membrane-bound lipase of the mutant

Mutant cells (7.5-liter culture, about 25 g wet weight) were grown and harvested, and the lipase was partially purified as described under "Experimental Procedures."

Step	Specific activity	Purification	Yield
		-fold	%
Protoplasts	0.025	1	(100)
A. Membranes	0.12	5	36
B. Base-washed membranes	0.13	5.2	27
C. Urea-supernatant	0.56	22	8.5
D. DEAE-cellulose eluate	3.7	148	2.8

Polyacrylamide gel electrophoresis of membranes, the urea supernatant, and the DEAE-cellulose eluate was performed by a modification of the method of Weber and Osborn (3, 24). The gels showed that the final preparation was substantially purified but still contained several protein bands (data not shown).

The solubilized lipase activity decayed rapidly in distilled water. It lost about half of its activity in 3 to 4 days when stored in 0.05 M sodium phosphate, pH 7.0, at 0°C. Its stability was not affected by 0.1 M NaCl. It was less stable in 0.001 M $CaCl_2$ and was inhibited by glycerol. Attempts to concentrate the enzyme for further purification resulted in its inactivation. The enzyme was stable at −20°C for 4 to 6 weeks.

Preparation of the Lipase from Growth Medium of the Wild Type—The extracellular lipase was prepared from a wild type culture as described under "Experimental Procedures." Its activity also decayed slowly at 0°C. Addition of

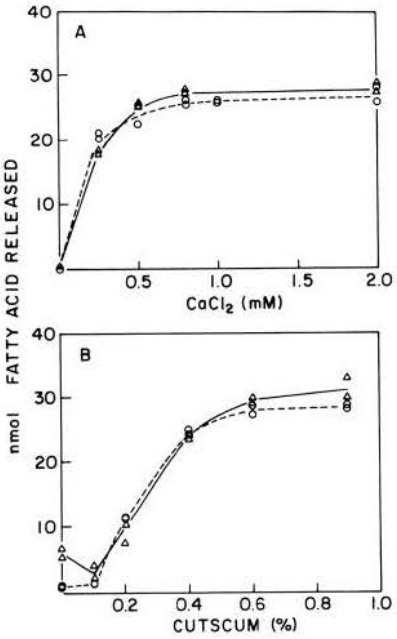

FIG. 3. Dependence of activity of the membrane-bound and extracellular lipases on $CaCl_2$ and detergent. The lipase from membranes of the mutant, solubilized and partially purified as described in Table II, and the lipase in concentrated growth medium of the wild type, prepared as described under "Experimental Procedures," were assayed under standard assay conditions except for the variable parameter. Each assay contained 0.003 units of lipase activity and was carried out for 10 min. *A*, dependence on $CaCl_2$. Assays of lipase from the wild type contained 0.12 mM EGTA to chelate the Ca^{2+} present in the growth medium. *B*, dependence on detergent. For these assays, the substrate was dispersed in 0.02% Cutscum. Detergent was added in increasing concentration to the assay mixture. The enzyme was added to each tube immediately preceding addition of substrate. △——△, membrane lipase; ○- - -○, extracellular lipase.

α-toluenesulfonyl fluoride, which inhibits some protease activities, improved its stability.

Dithiothreitol, lysophosphatidylethanolamine, valeric acid (0.01 and 0.1 mM), 2 mg/ml of ovalbumin, 0.1 and 0.5% cutscum, and storage at pH 9.0 or pH 5.0 had no effect on the stability of the enzyme. Valeric acid (6 mM), 0.01 M 2-mercaptoethanol, 0.4 M KCl, 0.1% deoxycholate, and glycerol destabilized the activity. The preparation was stable frozen at −20°C for at least 4 to 6 weeks.

Comparison of Membrane-bound and Extracellular Lipases—Several properties of the membrane-bound and extracellular lipases were compared. The optimal assay conditions for each lipase were identical (Figs. 3 and 4). The substrate specificities of the two lipases were similar, as well. In Fig. 5 is shown the dependence of extracellular and membrane-bound lipase activities on the concentration of phosphatidylethanolamine. Values of the apparent Michaelis constants for several other substrates are given in Table III.

The velocities of hydrolysis of each of the substrates were less than predicted by Michaelis-Menten kinetics at lipid concentrations above the apparent K_m, as illustrated in Fig. 5 for phosphatidylethanolamine. Thus, both enzymes were inhibited by high concentrations of substrate.

In previous investigations from this laboratory it was found that the lipase of the mutant had the positional specificity of a phospholipase A_1 (2). However, the results in Table III show that the enzyme is not specific for phospholipids.

In order to determine whether the phospholipase and lipase activities reside in a single enzyme or two separate ones, the ratios of these activities were compared at different stages of

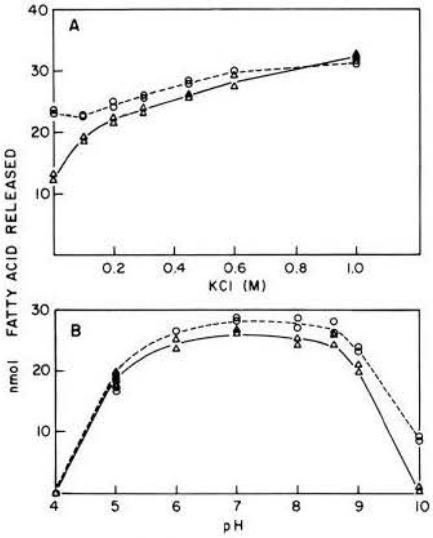

FIG. 4. Dependence of activity of the membrane-bound and extracellular lipases on salt concentration and pH. The lipases from mutant and wild type, prepared as described in Fig. 3, were assayed under standard assay conditions except for the variable parameter. Each assay contained 0.003 unit of lipase activity and was carried out for 10 min. *A*, dependence on salt concentration. *B*, dependence on pH. The buffers used were: sodium acetate, pH 4.0, pH 5.0; Tris/maleate, pH 5.0, pH 6.0, pH 7.0, pH 8.0; glycine, pH 8.6, pH 9.0, pH 10.0. Each buffer was present at a final concentration of 0.05 M in the assay mixture. △——△, membrane lipase; ○- - -○, extracellular lipase.

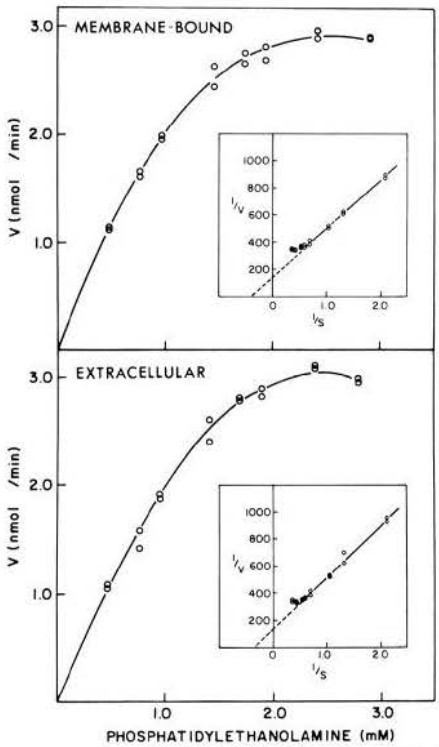

FIG. 5. Dependence of activity of the partially purified membrane lipase and the lipase from growth medium on the concentration of phosphatidylethanolamine from *E. coli*. *Top,* lipase from membranes of the mutant, solubilized and partially purified as described in Table II (DEAE-cellulose eluate), was assayed under the standard assay conditions except for the variable parameter. Each assay contained 0.003 unit of lipase activity and was carried out for 10 min. [1-*acyl*-^{3}H]Phosphatidylethanolamine was prepared as described under "Experimental Procedures." *Bottom,* lipase from the concentrated growth medium of the wild type, prepared as described under "Experimental Procedures," was assayed as described in above. The ordinate scale of the insets is [micromole/min]$^{-1}$.

TABLE III

Substrate specificity of the solubilized lipase from mutant membranes and the lipase from wild type growth medium

Values for the apparent K_m and the apparent V_{max} for each substrate were obtained from double-reciprocal plots of data obtained as described in Fig. 5. After excluding points at which lipase activity was inhibited by substrate, the best straight line fit through each set of points was determined by the method of least squares.

	Apparent K_m		Apparent V_{max}	
	Membrane-bound	Extracellular	Membrane-bound	Extracellular
	mM		*nmol/min*	
E. coli phosphatidylethanolamine	2.7	2.5	7.2	6.9
B. subtilis phosphatidylethanolamine	1.8	1.4	5.3	5.6
B. subtilis phosphatidylglycerol	4.4	9.5	11.	22.
B. subtilis diphosphatidylglycerol	1.4	1.1	0.5	0.6
B. subtilis diglyceride	1.9	1.9	3.2	3.2
B. subtilis diglucosyl diglyceride	1.7	3.0	2.9	3.4

purification of both the membrane-bound and extracellular enzymes. The ratio was calculated by dividing the velocity of hydrolysis of *E. coli* phosphatidylethanolamine (at a concentration of 2.3 mM) by the velocity of hydrolysis of 1,2-diglyceride (at a concentration of 1.3 mM). This ratio was 2.4 for the membrane-bound enzyme before its solubilization and 2.5 after its solubilization and purification. Both crude and partially purified extracellular lipase had a ratio of 2.7. The similarity of this ratio before and after purification of the enzymes indicates that the lipase and phospholipase activities co-purified and, thus, probably reside in the same molecule. Another observation in favor of this conclusion was that both the lipase and phospholipase activities were abolished by the inhibitor protein from the wild type.

Both the membrane-bound and extracellular lipases were inactivated by the inhibitor protein from the wild type (Fig. 6). The time courses of their inactivation were identical (Fig. 6*B*). The membrane lipase was slightly more sensitive to the inhibitor than was the lipase from growth medium (Fig. 6*A*). This could have been due either to the greater purity of the membrane lipase or to a slight difference in the structure of the two molecules. No other lipase that was tested was affected by the inhibitor, including the Ca^{2+}-independent phospholipase that appears in *B. subtilis* during sporulation (4). Consequently, the sensitivity of the extracellular lipase to the inhibitor protein suggests rather strongly that it is related to the membrane lipase.

Release of the Membrane-bound Lipase into the Growth Medium—The strong similarity between the membrane-bound and extracellular forms of the lipase indicated that the membrane-bound form might be an intermediate in the secretion of the lipase. In order to test this hypothesis, we searched for conditions under which we would be able to block formation of new membrane-bound lipase and show release of the remaining cell-bound enzyme into the growth medium. Such an experiment is shown in Fig. 7. Mutant cells were harvested in late logarithmic phase, resuspended in fresh prewarmed medium, and aerated. The optical densities of the cultures continued to increase, indicating that the cells were still dividing. Under these conditions, lipase continued to be secreted for about 1 h. During the same time, unexpectedly, membrane-bound enzyme was lost from protoplasts that were prepared from the cells. About 60% of the lipase activity was lost from the membranes, and an equivalent amount appeared in the medium. Similar results were obtained when the cells were resuspended in medium containing chloramphenicol, except that under these conditions cell growth was arrested. If the resuspended cells were not aerated, no lipase disappeared from the membranes, and no lipase appeared in the growth medium (data not shown). These experiments support the hypothesis that the membrane lipase is an intermediate in the secretion of extracellular lipase.

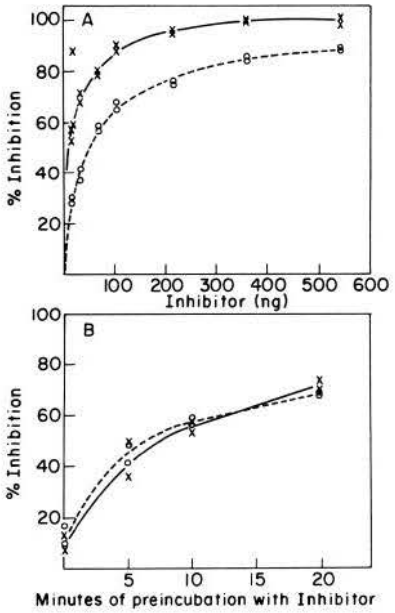

FIG. 6. Dependence of inhibition of the lipases from mutant and wild type on inhibitor protein concentration and time. *A*, varying amounts of purified lipase inhibitor (HA2 fraction, kindly provided by Dr. S. S. Krag (3)), diluted with a solution of ovalbumin (0.1 mg/ml) in distilled water, were preincubated with 0.8 μg of the solubilized and partially purified membrane-bound lipase from the mutant, or 56 μg of partially purified lipase from growth medium of the wild type (see "Experimental Procedures") for 10 min at 30°C in 0.070 ml of 1.4 mM $CaCl_2$. A 2-min lipase assay was initiated by the addition of 230 nmol of [1-*acyl*-^{3}H]phosphatidylethanolamine (1400 cpm/nmol) which had been dispersed by sonication in 2% Cutscum and 0.167 M Tris/maleate, pH 8.0. The final concentration of the components in the assay mixture were the standard concentrations described under "Experimental Procedures." The reaction was terminated, and the ^{3}H-fatty acids were extracted as described under "Experimental Procedures." Per cent inhibition was calculated as previously described (3). *B*, solubilized and partially purified lipase from the mutant (0.8 μg) was incubated with 18 ng of purified inhibitor for varying amounts of time, then a 2-min lipase assay was initiated as described in *A*. Similarly, 72 ng of inhibitor was incubated with 56 μg of partially purified lipase from the growth medium of the wild type. Per cent inhibition was calculated as previously described (3). ×——×, membrane lipase. ○- - -○, extracellular lipase.

Subcellular Distribution of the Membrane-bound Lipase—The *Bacilli* contain two types of membrane: plasma membrane, which surrounds the cytoplasm, and mesosomal membrane, which is found in small vesicles located in clusters in the space between the cell wall and the plasma membrane. The presence of 60% of the cell-bound form of penicillinase in

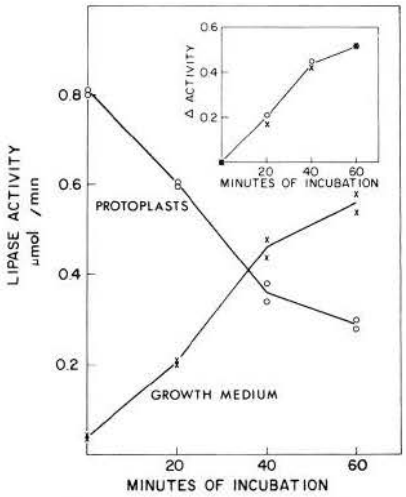

FIG. 7. Release of cell-bound lipase into growth medium. A 500-ml culture of mutant cells was grown to an optical density of 2.8, as described under "Experimental Procedures." Cells were harvested at 25°C from 40-ml aliquots of the culture. Each cell pellet was resuspended in 50 ml of fresh, prewarmed (30°C), aerated medium. The reconstituted cultures were transferred to prewarmed flasks and incubated with shaking at 30°C each for one of the times indicated on the *abscissa*. At the end of each incubation period, the optical density of the culture was measured, then the flask was cooled to 0°C, and 50 μl of α-toluenesulfonyl fluoride was added. The optical density at 0 min was 2.8, at 20 min, 3.1, at 40 min, 3.2 at 60 min, 3.6. Cells were harvested and converted to protoplasts as described under "Experimental Procedures." Lipase activity in growth medium and in protoplasts was determined as described under "Experimental Procedures"; ×——×, growth medium; ○——○, protoplasts. The *inset* shows the change in lipase activity from 0 min in growth medium and protoplasts. The experiment was performed twice with similar results.

the mesosomal vesicles of *B. licheniformis* led Ghosh *et al.* (25) and Sargent *et al.* (26) to propose that mesosomes are involved in the secretion of extracellular penicillinase by this organism.

Because of the likelihood that the membrane-bound lipase of our mutant was a secretion intermediate, we investigated its subcellular distribution. Four subcellular fractions were analyzed: "periplasmic" molecules (soluble molecules located between the cell wall and the cytoplasmic membrane), mesosomal vesicles, cytosol, and cytoplasmic membranes. The cells were fractionated as described under "Experimental Procedures." EGTA was present in the first steps to inhibit lipase activity and thus prevent lysis of the protoplasts and fragmentation of the membranes during removal of the cell wall. Recovery of lipase activity in such experiments was about 80%. Most of the lipase activity (50 to 70%) was associated with the membrane fractions. A variable amount of lipase was recovered in the cytosol (20 to 30%), depending on the conditions during lysis of the protoplasts. Recovery of some lipase in the cytosol suggested that the lipase is a peripheral membrane protein (27). The fact that it was removed from membranes by 3 M urea is consistent with this interpretation. Only a small portion of lipase activity (8% or less) was recovered in the periplasmic space and less than 1% was associated with the mesosomal vesicles. To strengthen the conclusion that little or no membrane-bound lipase was associated with mesosomal vesicles, we examined the purity of the cytoplasmic and mesosomal membrane fractions using electron microscopy, and the marker enzyme, succinate dehydrogenase, which is 30 times more concentrated in the cytoplasmic membrane than in mesosomes (13). Electron micrographs of the cytoplasmic membrane fraction showed a predominance of

TABLE IV

Lipase activity in membrane fractions of the mutant

Mutant cells were grown, harvested, and fractionated as described under "Experimental Procedures," with the following modifications. Pellet 1 was resuspended in 4 ml of sucrose buffer containing 0.01 M EGTA and 0.025 M $MgCl_2$. The suspension was added dropwise with stirring to 120 ml of Mg^{2+}/phosphate buffer containing 1.2 mg of DNase and 1.3 mg of RNase. Each of the membrane fractions was washed once with Mg^{2+}/phosphate buffer, before the 0.01 M phosphate buffer wash. The membrane fractions were analyzed for protein, succinate dehydrogenase activity, and lipase activity as described under "Experimental Procedures."

Fraction	Protein	Succinate dehydrogenase[a]		Lipase[a]	
		Specific activity	Total activity	Specific activity	Total activity
	mg				
Cytoplasmic membranes	22.5	0.44	9.9	0.05	1.1
Mesosomal membranes	1.5	0.051	0.07	0.007	0.01

[a] One unit of activity is defined as the amount of enzyme catalyzing formation of 1 μmol of product/min.

TABLE V

Inhibitor activity in membrane fractions of the wild type

Wild type cells were grown, harvested, and fractionated as described under "Experimental Procedures." The purified cytoplasmic and mesosomal membrane pellets were analyzed for protein, succinate dehydrogenase activity, and inhibitor activity as described under "Experimental Procedures." The total recovery of protein after density gradient fractionation of the crude cytoplasmic membrane pellet was 44%, the recovery of succinate dehydrogenase activity was 61%, and the recovery of inhibitor activity was 68%. The corresponding recoveries after fractionation of the crude mesosomal pellet were 38, 70, and 63%, respectively.

Fraction	Protein	Succinate dehydrogenase[a]		Inhibitor[b]	
		Specific activity	Total activity	Specific activity	Total activity
	mg				
Cytoplasmic membranes	35	1.0	36	360	12,600
Mesosomal membranes	8.7	0.021	0.18	610	5,300

[a] One unit of enzyme activity is defined as the amount of enzyme catalyzing formation of 1 μmol of product/min.

[b] One unit of inhibitor activity is defined as the amount necessary to give 30% inhibition of 0.0013 units of membrane-bound lipase in a standard 20-min assay.

the large membranous profiles, while micrographs of the mesosomal fraction showed mostly small vesicles. The micrographs revealed some cross-contamination between the two fractions; nevertheless, the specific activity of succinate dehydrogenase was 8.6 times higher in the cytoplasmic membrane fraction than in the mesosomes (Table IV). The distribution of lipase activity shown in Table IV was almost identical to that of the marker enzyme. The data indicated that the lipase activity in the mesosomal fraction was due largely to contamination by pieces of cytoplasmic membrane.

In a similar experiment, the subcellular location of the membrane-bound inhibitor in the wild type was investigated. Wild type cells were fractionated by the method of Ferrandes *et al.* (13) described under "Experimental Procedures." This method includes a sucrose gradient step which eliminates most of the cross-contamination between the two membrane fractions. Electron micrographs of the cytoplasmic and mesosomal membrane fractions showed very little cross-contamination. The specific activity of succinate dehydrogenase (Ta-

ble V) was 48 times higher in the cytoplasmic membranes than in the mesosomes, indicating that the cytoplasmic membranes were highly purified. Nevertheless, the specific activity of the inhibitor in the mesosomal membranes was only twice that in the cytoplasmic membranes. These results show that the membrane-bound inhibitor is located in both membrane fractions, but has a higher specific activity in the mesosomes.

DISCUSSION

Earlier reports from this laboratory described a mutant of *B. subtilis,* called CMK_{33}, that hydrolyzed its membrane lipids during enzymatic digestion of the cell wall (1, 2). The lipid was hydrolyzed by a membrane-bound lipase that was present in protoplasts of the mutant but was not detectable in the wild type. A specific protein inhibitor of the lipase was found in wild type cells and was absent in the mutant. This paper reports that both mutant and wild type cells secrete an extracellular lipase that is closely related to the membrane-bound lipase of the mutant. Production of both enzymes is stimulated by a high molecular weight component of the growth medium. No significant differences were found between the optimal assay conditions and substrate specificities of the two enzymes. This similarity suggests that the membrane-bound lipase may be an intermediate in the secretion of the extracellular lipase.

This hypothesis was supported by an experiment in which membrane-bound lipase was apparently transferred from the membrane into the extracellular medium. When mutant cells were harvested in late logarithmic phase and resuspended in fresh medium, they continued to secrete lipase for about 1 h. During this time, the appearance of new extracellular lipase activity in the culture medium was paralleled by an equivalent decrease in the amount of membrane-bound lipase activity found in protoplasts prepared from the cells. It is not clear why the shift to fresh medium apparently inhibited the formation of new membrane-bound lipase. It may be that synthesis of exoenzyme is more easily disrupted than general protein synthesis, or that a factor that builds up in growth medium is required for lipase synthesis. The results shown in Fig. 2 and discussed in the text suggest that secretion of exocellular enzyme stops in stationary phase. It may be that cessation of synthesis of membrane lipase by the mutant occurs before cessation of secretion and that the cells were shifted to new medium at a time between these two events. It could also be that both processes had stopped at the time of the shift, but that secretion of the lipase resumed under the new conditions more quickly than lipase synthesis. Cells that were harvested and resuspended in the same way, but were not aerated, did not secrete lipase and did not lose the membrane-bound activity. Addition of chloramphenicol to the resuspended cultures inhibited growth of the cultures but did not interfere with the appearance of new lipase in growth medium or with the disappearance of membrane-bound lipase. This indicated that *de novo* protein synthesis was not required for secretion of lipase under these conditions and strengthened the hypothesis that preformed membrane enzyme was the source of the newly secreted lipase.

If the membrane lipase is in fact a secretion intermediate and does not have a function within the cells themselves, then the role of the protein inhibitor found in the wild type is probably to protect the membranes of the cell from the lipase during and after its secretion. This would explain why lipase activity has never been detected in more than trace amounts in wild type cells. An analogous intracellular protein inhibitor of ribonuclease has been reported in *Bacillus amyloliquefaciens* (28). Presumably, the function of this protein is to protect cellular ribonucleic acid from hydrolysis by the ribonuclease.

Several recent studies have provided evidence that extracellular proteins are synthesized on membrane-bound ribosomes, and are extruded through the membrane during their synthesis (29, 30). In some cases, it has been shown that excreted proteins that are synthesized *in vitro* contain an extra hydrophobic NH_2-terminal sequence. It is postulated that this sequence acts as a signal that results in the attachment of the ribosome to the membrane in such a way that synthesis of the nascent chain proceeds vectorially, and the completed protein emerges on the other side of the membrane (31, 32). In eukaryotes, a processing enzyme which removes the hydrophobic sequence from light chains was demonstrated in the membranes of rough microsomes (31). Inouye and Beckwith (32) found evidence for a similar kind of processing of the periplasmic protein, alkaline phosphatase, in *E. coli.* They showed that the protein that was synthesized *in vitro* from the alkaline phosphatase message, contained a hydrophobic sequence that was not present in extracellular alkaline phosphatase. This sequence was removed by an enzyme activity that was associated with the outer membrane.

There is some evidence for a similar secretion mechanism in Gram-positive bacteria (33–35). Lampen and co-workers have studied the secretion of penicillinase by *Bacillus licheniformis.* About half of this enzyme is extracellular and the other half is cell-bound. The cell-bound enzyme was found to be 25 amino acid residues longer than the extracellular one and contained a hydrophobic NH_2 terminus, phosphatidylserine (36, 37). Nagata *et al.* (38) have described three cell-bound isozymes of the exoenzyme, α-amylase, in *B. subtilis* Marburg (from which CMK_{33} is derived). One of these isozymes had the same molecular weight as the exoenzyme; the other two had larger molecular weights, as determined by gel chromatography. All three, as well as the exoenzyme, were coded by the same gene. In contrast with penicillinase, and with the lipase in this paper, the cell-bound forms of α-amylase were only 1 to 5% of the total α-amylase in a given culture.

The observation of a membrane-bound precursor of the extracellular lipase in CMK_{33} is consistent with the secretion mechanism suggested by Blobel and others (29–32). However, because both the exoenzyme and the solubilized membrane enzyme were excluded from Sephadex G-200 (data not shown), we do not yet know if the polypeptide chains of the two enzyme forms differ in molecular weight.

It seems clear, nevertheless, that some form of processing of the membrane-bound lipase occurs before it is released. An indication of this is that the lipase activity is expressed in mutant membranes only during and after digestion of the cell wall (2). If the lipase were active in intact mutant cells, the cells would be expected to show an increased turnover of fatty acids. Numerous attempts to demonstrate such an increased turnover in mutant cells were unsuccessful (4). Even when the cells were treated with arsenate to inhibit ATP formation and, consequently, prevent reactivation of fatty acids that might be released by the lipase, there was no buildup of free fatty acids. These results indicate that the lipase is either absent or inactive in intact cells. There are two possible explanations for the appearance of membrane-bound lipase activity only during and after digestion of the cell wall. One is that a structure within the cell wall is necessary for the release of newly synthesized lipase into the extracellular medium. If this is the case, then lipase that is synthesized while the wall is being degraded might be inserted through the membrane and remain there, because the machinery necessary for the release of the enzyme has been disrupted. However, Sanders and May

(39) and Sargent *et al.* (40) showed that protoplasts of Bacilli can actively secrete three different enzymes. This indicates that an intact cell wall is not necessary for the release of any of these enzymes. Another possible explanation is that the lipase normally remains in an inactive state while it is associated with the membrane; then during its secretion it is activated by an enzyme located in the cell wall. If this is the case, digestion of the cell wall might free this endogenous activating enzyme and allow it to come into contact with and activate the lipase while it is still in the cytoplasmic membrane. That the processing of extracellular enzymes can in fact occur in the cell wall rather than in the cytoplasmic membrane is suggested by the results of Inouye and Beckwith (32) who described an alkaline phosphatase processing activity in the outer membrane fraction of *E. coli* cells.

Sargent and co-workers have suggested that mesosomal vesicles may play a role in exoenzyme secretion (25, 26). They reported that 60% of the cell-bound form of penicillinase in *B. licheniformis* was associated with mesosomal vesicles. In addition, they showed that the mesosomal penicillinase was the precursor of exoenzyme that was released when protein synthesis was inhibited by chloramphenicol. Our results show that there is very little lipase associated with mesosomes of CMK_{33}; consequently, it is unlikely that these structures are involved in secretion of lipase by *B. subtilis*. However, the distribution of lipase inhibitor between mesosomal and cytoplasmic membranes in the wild type is similar to the distribution of cell-bound penicillinase in *B. licheniformis;* the inhibitor is present in both mesosomal and cytoplasmic membranes. The presence of inhibitor in both types of membrane would be expected if its function were to protect membranes from external lipase or from lipase that might be free in the region between the cytoplasmic membrane and the cell wall during the process of lipase secretion.

From the studies described in this report it is clear that protoplasts of the mutant, CMK_{33}, contain a membrane-bound lipase that is closely related to the extracellular lipase that is secreted by both mutant and wild type cells. Our hypothesis is that the membrane-bound lipase is an intermediate in the secretion of the extracellular lipase. Because the intermediate is accessible for study in the mutant, but not in the wild type, the mutant may be a useful tool in studies of the mechanism of secretion of exoenzymes by Bacilli.

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